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ACTIVATION MECHANISMS OF CLASS F RECEPTORS

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Activation mechanisms of Class F receptors

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Cover: Placing FZDs and G proteins together in the context of human physiology – from WNT-FZD_5-$G\alpha_q$ interaction at the plasma membrane in pancreatic duct cells to the pancreas itself; artwork by Jennifer M. Wright.

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ACTIVATION MECHANISMS OF CLASS F RECEPTORS
THESIS FOR DOCTORAL DEGREE (Ph.D.)

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To my mother and father.

ABSTRACT

Our genetic makeup is composed of thousands of genes that code for the proteins involved in all aspects of cellular function. Proteins are molecular machines that dynamically and structurally respond to stimuli. Cells are able to sense their environment and react accordingly through the help of receptors – a type of protein – embedded in the cellular membrane that binds extracellular molecules known as ligands. The innate ability of receptors to specifically interact with and influence structural changes in partner proteins forms the basis for signalling cascades that regulate cellular processes. In diseased individuals, genetic mutations can result in changes in the structure of proteins that will affect the tertiary and quaternary structures of a protein or proteins in complex. This can have drastic effects on protein function – ultimately compromising cellular fate.

The use of animal and plant-based products in modern medicine led to the development of pharmacology – the study of drug effects in living organisms. Receptor-labelling techniques and molecular cloning enabled us to establish that many of these molecules were acting on a family of proteins called G protein-coupled receptors (GPCRs). Our knowledge of the structural changes exerted on the receptor upon ligand binding has led to the development of new drugs with increased subtype specificity and fewer side effects.

While our knowledge related to prototypical GPCRs like adrenoreceptors and opioid receptors has flourished in recent years, their distantly related cousins – known as Frizzleds (FZDs) and Smoothened (SMO) – have suffered from an inability to assess their GPCR nature. FZDs interact with the WNT family of lipoglycoproteins and SMO is indirectly regulated by Hedgehog (Hh) to orchestrate important processes in embryonic development and adult homeostasis. Misregulation of WNT and Hh signalling leads to the pathogenesis of numerous diseases for which treatments are limited. With respect to WNT signalling, the lack of effective drugs targeting FZDs is in large part due to a knowledge gap resulting from current dogma that places emphasis on protein complex formation rather than the structural changes involved in protein activation.

The findings compiled in **papers I** to **III** provide structural insight into the mechanisms underlying FZD/SMO activation. A combination of genomic, biophysical and biochemical approaches was used to unravel the details surrounding FZD/SMO complex formation and the structural dynamics upon ligand binding that lead to full receptor activation and downstream signalling. This has implications for the development of effective treatments for diseases where FZDs and SMO can be targeted. Characterizing the conformational landscape of these receptors will enable us to drive drug discovery efforts forward.

LIST OF SCIENTIFIC PAPERS

I. Julian Petersen*, **Shane C. Wright***, David Rodríguez, Pierre Matricon, Noa Lahav, Aviv Vromen, Assaf Friedler, Johan Strömqvist, Stefan Wennmalm, Jens Carlsson and Gunnar Schulte. Agonist-induced dimer dissociation as a macromolecular step in G protein coupled receptor signaling. *Nat. Commun.* **8**, 226 (2017).

II. **Shane C. Wright***, Maria Consuelo Alonso Cañizal*, Tobias Benkel, Katharina Simon, Christian Le Gouill, Pierre Matricon, Yoon Namkung, Viktoria Lukasheva, Gabriele M. König, Stéphane A. Laporte, Jens Carlsson, Evi Kostenis, Michel Bouvier, Gunnar Schulte‡ and Carsten Hoffmann‡. FZD_5 is a $G\alpha_q$-coupled receptor that exhibits the functional hallmarks of prototypical GPCRs. *Sci. Signal*. **11,** eaar5536 (2018).

III. **Shane C. Wright***, Paweł Kozielewicz*, Maria Kowalski-Jahn, Julian Petersen, Carl-Fredrik Bowin,Greg Slodkowicz, Maria Marti-Solano, David Rodríguez, Belma Hot, Najeah Okashah, Katerina Strakova, Jana Valnohova, M. Madan Babu, Nevin A. Lambert, Jens Carlsson and Gunnar Schulte. A conserved molecular switch in TM6/7 of Class F receptors regulates receptor activation and pathway selection. *Manuscript*.

* These authors contributed equally.

ADDITIONAL PUBLICATIONS

I. Gunnar Schulte and **Shane C. Wright**. Frizzleds as GPCRs – More conventional than we thought! *Trends Pharmacol. Sci.* **39**, 8 (2018).

CONTENTS

LIST OF ABBREVIATIONS

7TM	seven-transmembrane-spanning
APC	adenomatous polyposis coli
BRET	bioluminescence resonance energy transfer
CD	circular dichroism
CELSR	cadherin EGF LAG seven-pass G-type receptor
CFP	cyan fluorescent protein
CK1	casein kinase 1
Cos2	Costal-2
CRD	cysteine-rich domain
CRISPR	clustered regularly interspaced short palindromic repeats
DAAM	Dishevelled-associated activator of morphogenesis
DAG	diacylglycerol
dcFRAP	double color fluorescence recovery after photobleaching
DDM	*n*-Dodecyl β-D-maltoside
DIX	Dishevelled-Axin
DVL	Dishevelled
ECD	extracellular domain
ECL	extracellular loop
ERK1/2	extracellular signal-regulated kinase 1/2
Fc	Fragment crystallizable region
FCCS	fluorescence cross correlation spectroscopy
FCS	fluorescence correlation spectroscopy
FEVR	familial exudative vitreoretinopathy
FlAsH	fluorescein arsenic hairpin binder
FRET	fluorescence resonance energy transfer
FZD	Frizzled
GEF	guanine nucleotide exchange factor
GFP	green fluorescent protein
Gli	glioma-associated oncogene
GPCR	G protein-coupled receptor

GRK	G protein-coupled receptor kinase
GSK3	glycogen synthase kinase 3
HDL	high-density lipoprotein
Hh	Hedgehog
HNF-3β	hepatocyte nuclear factor-3β
ICL	intracellular loop
ITC	isothermal titration calorimetry
LRP	low-density lipoprotein-receptor-related protein
MAPK	mitogen-activated protein kinase
mG	mini G protein
MLE-12	mouse lung epithelial cells
MMTV	mouse mammary tumor virus
PCP	planar cell polarity
PDAC	pancreatic ductal adenocarcinoma
PDZ	PSD95/Dlg1/zo-1
PKA	protein kinase A
PKC	protein kinase C
PLC	phospholipase C
PORCN	Porcupine
PPI	protein-protein interaction
PTCH	Patched
PTK7	Tyrosine-protein kinase-like 7
PTX	Pertussis toxin
RhoA	Ras homolog gene family, member A
RNF-43	ring finger protein 43
ROR	receptor tyrosine kinase-like orphan receptor
RYK	receptor tyrosine kinase
SAG	Smoothened agonist
SEC	size exclusion chromatography
SMO	Smoothened
TCF/LEF	transcription factors T cell factor/ lymphoid enhancer factor

TIRF-M	total internal reflection fluorescence microscopy
TM	transmembrane
TSS	transcription start site
VANGL	Van Gogh-like protein
WNT	Wingless/int-1

1 INTRODUCTION

More than 100 years ago, the first sex-linked mutation was discovered in *Drosophila melanogaster* that would serve as the basis for a flurry of genetic studies based on phenotypic screening (Morgan, 1910). More than half a century later, efforts to dissect the genes involved in segment polarity converged on wingless flies and short larvae with spiky cuticles (Sharma and Chopra, 1976; Wieschaus et al., 1984). These phenotypes were found to be the result of misregulation in the WNT and Hedgehog (Hh) pathways respectively – signalling pathways that direct embryonic development and are involved in pathophysiology.

1.1 WNT SIGNALLING

The gene product that would later be identified as WNT was originally discovered through a hypomorphic mutation in the wg^{1} allele (Sharma and Chopra, 1976). Studies on the mouse mammary tumor virus (MMTV) revealed the insertion/integration of proviral DNA into a gene that was appropriately called *int-1* (Nusse and Varmus, 1982). However, it was later shown that *wg* and *int-1* were identical leading to the current nomenclature for the gene family – WNT (wingless-related integration site) (Nusse et al., 1991; Rijsewijk et al., 1987). Analysis of the gene product and further biochemical validation revealed that WNTs were secreted proteins. The finding that WNT was a morphogen involved in cell-to-cell communication led to the hypothesis that there must be a transmembrane receptor transducing these signals. Cells expressing Dfz2 were able to bind Wg suggesting that Frizzled proteins were the receptors for WNT molecules (Bhanot et al., 1996). In *Drosophila*, there are seven *WNT* and four *FZD* genes (Wu and Nusse, 2002). In mammals, this number is scaled up to 19 *WNT* and 10 *FZD* genes (Schulte, 2010). The specificity of these interactions has yet to be completely elucidated especially given the fact that redundancy exists among WNT-FZD pairs and the inherent difficulties in assessing interactions between full-length FZD and purified WNT – not to mention the potential involvement of co-receptors (Dijksterhuis et al., 2015; Eubelen et al., 2018; Wu and Nusse, 2002).

The overlap in phenotypes linked to segment polarity in *Drosophila* and the inability to decipher the linearity of signalling pathways and the gene products involved therein provoked the use of biochemical assays to identify the composition of functional protein complexes.

1.1.1 WNT/β-catenin signalling

Armadillo – the *Drosophila* equivalent of the intracellular signal transducer β-catenin – was found to accumulate in regions where Wg/WNT was expressed in the developing fly embryo (Riggleman et al., 1990). In line with this observation, embryos harbouring mutations in *armadillo* were found to have the same defect in segmentation of the body plan as *wg*, *porcupine* and *dishevelled* (DVL) (Clevers, 2006; Theisen et al., 1994). Later, it was discovered that a single-pass transmembrane protein called arrow in *Drosophila* or low-density lipoprotein-receptor-related protein (LRP) in vertebrates formed a complex with Frizzled proteins to transduce Wg signals across the membrane (Wehrli et al., 2000). With the

identification of mutations in the *Drosophila* gene *shaggy/zeste-white 3* – known as glycogen synthase kinase 3 (GSK3) in vertebrates – a regulatory component was implicated in segment polarity (Peifer et al., 1994). Soon thereafter, the mutants that shared similar phenotypes were found to constitute a new signalling pathway – the WNT/β-catenin pathway.

This pathway has been reviewed extensively by others (Clevers, 2006; Grainger and Willert, 2018; Nusse and Clevers, 2017; Steinhart and Angers, 2018) and will only briefly be described here. It should be noted, however, that specific WNTs (e.g. WNT-1 and WNT-3A) and FZDs (e.g. FZD_4 and FZD_5) display a higher propensity to initiate this signalling cascade. In the absence of WNT, β-catenin is part of the cytosolic "destruction complex" with Axin, adenomatous polyposis coli (APC), GSK3 and CK1. Axin acts as a scaffold to facilitate phosphorylation of β-catenin by GSK3 and CK1. Phosphorylated β-catenin is then ubiquitinated by E3 ubiquitin ligases and targeted for proteasomal degradation. When WNT binds FZD/LRP, a signalosome is thought to form, allowing for FZD-bound DVL and LRP to become phosphorylated. Phosphorylation of LRP is carried out by GSK3 and CK1 leading to the recruitment of Axin from the "destruction complex." This promotes the stabilization of β-catenin and its translocation to the nucleus where it acts as a transcriptional co-activator with the transcription factors T cell factor/ lymphoid enhancer factor (TCF/LEF).

To date, slightly over half of the 19 WNTs are available for purchase as recombinant protein albeit with unknown specific activity and antibodies with sufficient isoform selectivity do not exist to be able to detect and distinguish heterologous expression in cells. This has hampered efforts to exhaustively characterize which WNTs are activating the β-catenin pathway. An added level of complexity comes from the finding that other proteins at the interface of WNT/FZD signalling may be required for certain WNT paralogues to achieve signal specification (Eubelen et al., 2018). However, WNTs are not the only ligands that bind to FZDs and initiate β-catenin signalling. Investigation into retinal vascularization led to the discovery of a molecular oddity. The similarity in vascular phenotypes exhibited by patients with either Norrie disease or familial exudative vitreoretinopathy (FEVR) suggested that the respective disease-associated variants, Norrin and FZD_4 respectively, were a ligand-receptor pair (Xu et al., 2004). Moreover, the signalling outcome of Norrin binding to FZD_4 was found to be activation of the β-catenin pathway. The diversity in ligand-FZD pairs, not only within the WNT family of lipoglycoproteins, but extending to other secreted proteins of unique protein structures reiterates the notion that there remains a large knowledge gap in our understanding of how FZDs or FZD-containing complexes transduce extracellular signals.

1.1.2 β-catenin-independent signalling

Classification of the different WNT signalling branches was originally based on studies in *Drosophila*, *Xenopus* and zebrafish of which two β-catenin-independent pathways were proposed: WNT/planar cell polarity (PCP) and WNT/Ca^{2+} (Niehrs, 2012). The number of components found to participate in these pathways has grown substantially in recent years. In

general, pathway selectivity is thought to be dependent on specific co-receptors, WNTs and higher order complex formation.

In the case of PCP, asymmetry is established through molecular complexing on adjacent cells. Briefly, FZD forms complexes with cadherin EGF LAG seven-pass G-type receptor (CELSR) and WNT co-receptors like receptor tyrosine kinase-like orphan receptor (ROR) or receptor tyrosine kinase (RYK). CELSR forms homotypic interactions in *cis* associating with FZD-DVL in one cell and Van Gogh-like protein (VANGL)-Prickle in the other (Chen et al., 2008). This intercellular complex can be destabilized through the dissociation of Prickle from VANGL (Ressurreição et al., 2018). Surface expressed and stabilized FZD binds WNT mediating the activation of the small GTPase Ras homolog gene family, member A (RhoA) – a process that requires DVL interaction with Dishevelled-associated activator of morphogenesis (DAAM) (Habas et al., 2001). The differential expression of these higher order complexes on opposing cells results in asymmetric cell division and cell fate determination.

The production of second messengers in the case of receptor ligands that cannot cross the plasma membrane triggers the activation of kinases that amplify signalling cascades. The production of cAMP or the mobilization of intracellular calcium are two second messenger systems that are intrinsically linked to GPCRs. Expression of WNT and FZD in zebrafish embryos led to the release of intracellular calcium which was blocked by inhibitors of inositol monophosphatase and pertussis toxin-sensitive G proteins (Slusarski et al., 1997). Because the release of Ca^{2+} is known to activate protein kinase C (PKC), follow-up studies tested whether WNT or FZD paralogues or the synergistic combination of both could lead to the recruitment of PKC to the plasma membrane and its subsequent activation. Not all WNTs or FZDs were capable of stimulating PKC reinforcing the idea that different signalling pathways existed downstream of specific WNT-FZD pairs (Sheldahl et al., 1999). During that period, it was well-known that DVL was involved in both WNT/β-catenin and WNT/PCP pathways albeit through different mechanisms – the Dishevelled-Axin (DIX) domain of DVL being required for the former, but not the latter. In a mechanism that was found to be both WNT- and G protein-independent, it was found that DVL was also able to mediate calcium mobilization in like manner to WNT/PCP signalling (Sheldahl et al., 2003). Although initially thought to be a distinct signalling pathway from WNT/PCP, WNT/Ca^{2+} has also been shown to regulate convergent extension suggesting that some overlap may exist between the two pathways.

There is a certain reluctance to designate the common denominator of WNT signalling. Even in the case of the WNT/β-catenin pathway, there is evidence for the involvement of G proteins (Katanaev et al., 2005; Liu et al., 2001). As for the β-catenin-independent signalling branches, the downstream proteins or second messengers that have been implicated therein have direct or indirect links to heterotrimeric G proteins. Although there is strong evidence for the involvement of DVL in all three branches of WNT signalling, it is not clear whether DVL facilitates G protein-mediated signalling or forms its own ternary complex with WNT and FZD to drive signal transduction.

1.2 HEDGEHOG SIGNALLING

Mutations in the genes encoding either Hh and Smoothened (SMO) resulted in a naked cuticle phenotype in *Drosophila* (Nüsslein-Volhard et al., 1984; Wieschaus et al., 1984). Around that time, it was thought that the cellular response to WNTs depended on the activity of Patched (PTCH) (Hidalgo and Ingham, 1990). PTCH is another protein involved in segment polarity that antagonizes Hh signals (Ingham et al., 1991). For a while, the interplay between these signalling molecules was not understood and it was thought that SMO might also be a receptor for Hh (Alcedo et al., 1996). Later studies revealed that PTCH was the receptor for Hh (three paralogues in vertebrates) and the molecular basis for this interaction was recently elucidated by cryo-EM (Gong et al., 2018; Qi et al., 2018). Like the WNT/β-catenin pathway, Hh signalling has been reviewed at length elsewhere (Arensdorf et al., 2016; Schulte, 2010). In short, when Hh is absent, PTCH represses SMO preventing glioma-associated oncogene (Gli) proteins from translocating to the nucleus to regulate gene transcription. Hh binds to PTCH relieving its inhibition of SMO. SMO is thought to be constitutively active and signal to Gli through unknown intracellular mediators to induce target gene expression. The mechanism through which PTCH controls SMO activity is still an area of active research. One hypothesis is that PTCH is a sterol transporter that regulates the availability of cholesterol for SMO – thought to act as an endogenous ligand for SMO activation (Gong et al., 2018; Taipale et al., 2002).

The mystery surrounding the endogenous ligand has also been an area of intense research and debate. Studies involving heterozygous *PTCH*$^{+/-}$ mice demonstrated an increased likelihood to develop medulloblastoma. Microarray analysis revealed an enrichment of transcripts involved in sterol synthesis. Further experimentation demonstrated that cholesterol and oxysterols could regulate SMO activity (Corcoran and Scott, 2006). Despite belonging to Class F, SMO does not bind WNTs (Wu and Nusse, 2002). Much like FZD_4, SMO has had a different evolutionary trajectory that is both similar and different to FZDs resulting in interaction with different ligands and/or higher constitutive activity. An interesting parallel between FZD and SMO ligands is the fact that WNTs are lipidated. It is attractive to assume that members of the Class F evolved to recognize nonpolar ligands via the CRD groove. However, this is clearly not the case for the FZD_4 ligand Norrin which is not lipidated and is predicted to engage two FZD_4 protomers simultaneously through an alternative CRD interface and molecular contacts with the linker domain (Bang et al., 2018; Shen et al., 2015). Another line of research suggests that while cholesterol and oxysterols may serve as ligands for SMO, it is not clear if they are allosteric modulators or true orthosteric agonists. In other words, it is not clear where the primary binding site of the endogenous SMO ligand is located. Non polar molecules like cholesterol have been shown to modulate the activity of other GPCRs in an allosteric manner much like ions (Dawaliby et al., 2016; Guixà-González et al., 2017).

The molecular intermediates that connect SMO activation to Gli in vertebrates are still a matter of debate. Based on the 7TM structure of SMO, heterotrimeric G proteins became a reasonable candidate. However, the physiological link between SMO and G proteins *in vivo* is controversial. Nevertheless, several studies have shown that SMO can functionally activate

heterotrimeric G proteins (Myers et al., 2017; Ogden et al., 2008; Riobo et al., 2006). Accepting SMO as a GPCR has been hindered by the translational aspect of these findings due to indications that pertussis toxin – an exotoxin inhibitor of $G\alpha_i$ – does not entirely prevent Hh signalling in different model organisms (Arensdorf et al., 2016; Philipp and Caron, 2009). This rationale fails to consider the possibility that SMO is capable of interacting with other heterotrimeric G proteins that are pertussis toxin-insensitive or that SMO can form ternary complexes with intracellular transducers other than G proteins to mediate Gli activation.

1.3 G PROTEIN-COUPLED RECEPTORS

The quest to understand how pharmacological agents work began at the turn of the 19th century with the Nobel Prize-winning work of Paul Ehrlich who famously stated in Latin, "*corpora non agunt nisi fixata*" or "a substance does not work unless it is bound" (Ehrlich, 1913). This was long before we knew that FZDs and SMO were receptors or that they belonged to the G protein-coupled receptor (GPCR) superfamily. It would take years before studies aimed at understanding the link between the enzyme adenylyl cyclase and hormones would ultimately bring this to fruition. Adenylyl cyclase catalyses the breakdown of ATP into the second messenger cAMP and pyrophosphate. Hormonal activation of adenylyl cyclase depends on the presence of GTP (Rodbell et al., 1971). Purification of this unknown regulatory component led to the identification of the first G protein (Northup et al., 1980). Meanwhile, chromatography experiments revealed that the G protein interacted with the β-adrenergic receptor in the presence of receptor agonist, but not antagonist (Limbird et al., 1980). Another piece of the puzzle was provided by the topographic model of the photoreceptor rhodopsin (Hargrave et al., 1984) – illustrating the conserved architecture consisting of seven hydrophobic helices that would become the foundation for the classification of proteins belonging to the GPCR superfamily.

1.3.1 Class F receptors

After the cloning of the gene for the β-adrenergic receptor (Dixon et al., 1986) and with the advent of human genome sequencing years later (Venter et al., 2001), we learned that there were more than 800 GPCR genes that were subsequently grouped into six classes based on phylogenetic analysis: Class A (rhodopsin-like), Class B (secretin receptor family), Class C (metabotropic glutamate), Class D (fungal mating pheromone receptors), Class E (cAMP receptors) and Class F (frizzled/smoothened) (Alexander et al., 2017). Consistent with other members of the GPCR superfamily, Class F receptors have the traditional 7TM architecture consisting of seven transmembrane-spanning α-helices connected by three extracellular (ECL) and intracellular loops (ICL) (**Fig. 1**). In mammals, there are eleven members: FZD_{1-10} and SMO. However, despite homologous topology, Class F receptors displayed a very different evolutionary history that was highly conserved across model organisms including worms, flies, sea quirts, fish and mammals in contrast to all other GPCR subfamilies (Schiöth and Fredriksson, 2005).

Structural differences in the GPCR superfamily begin to appear at the amino- and carboxy-terminal extremities. In contrast to Class A receptors, Class F receptors have an extracellular domain (ECD) similar to the receptors of Class B called a cysteine-rich domain (CRD). CRDs are also found in Class C receptors, secreted frizzled-related proteins (sFRPs) (Rattner et al., 1997) and several receptor tyrosine kinases (Masiakowski and Carroll, 1992) (the latter two of which are involved in WNT signalling). However, the ECD of Class C receptors is unusually large due to the adjacent Venus flytrap domain that serves as the orthosteric ligand binding site and is involved in receptor dimerization mediated by intermolecular disulfide bonds. Whereas the orthosteric binding site in Class A/B/C GPCRs is well-defined, further work is needed to understand the binding mode of the endogenous ligands for Class F receptors. In particular, our knowledge of the orthosteric binding site on FZDs comes from a crystal structure of recombinant CRD in complex with WNT (Janda et al., 2012). While this may be true, *in vivo* evidence suggests that the CRD is dispensable for WNT binding (Chen et al., 2004a; Lisovsky et al., 2002) – suggesting that there is more to the story. Moreover, methodological limitations have prevented the characterization of WNT affinity for full-length FZD. In Class B and F GPCRs, a linker connects the ECD to the transmembrane core and is presumed to play a role in the binding mode of the ligand either through orientation of the ECD relative to the core or contributions to the accessible surface area (Bang et al., 2018; Siu et al., 2013; Valnohova et al., 2018). On the opposite side of the membrane, the cytosolic tail of GPCRs varies in length – even within the Class F – and it is not clear to what degree the polypeptide sequence is helical or disordered. That being said, the C-terminal PSD95/Dlg1/zo-1 (PDZ) binding motif that is known to regulate GPCR signalling and trafficking is found among members of the Class F (Schulte, 2010). In particular, this domain mediates interactions with the scaffolding protein DVL via residues on all intracellular loops and the KTxxxW motif that precedes the PDZ binding motif – the latter being present in all FZDs, but not SMO. Interestingly, the parathyroid hormone receptor (PTHR – Class B) has a similar stretch of amino acids (KSxxxW) that also allows for interaction with DVL (Romero et al., 2010).

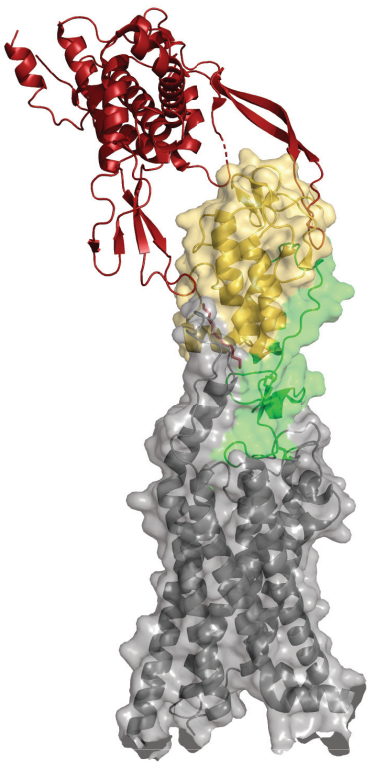

Fig. 1. Space-filling model of Class F receptor bound to WNT. *Xenopus* WNT-8 (red) (PDB ID: 4F0A) has been overlain with human SMO (PDB ID: 5V57). The CRD of SMO is depicted in yellow, the linker domain in green and 7TM domain in grey. The crystal structure of SMO was used for illustrative purposes to show how a WNT molecule bound to the CRD would appear relative to the core of a Class F receptor.

One of the major arguments against FZDs behaving as GPCRs has been the lack of conserved motifs with Class A receptors in the 7TM region (Angers and Moon, 2009). FZDs lack the DRY and NPxxY motifs that are proposed to be involved in receptor activation and G protein

coupling. Furthermore, Class F receptors do not possess the prototypical switches that regulate receptor activity; in particular, none of the 11 paralogues have an ionic lock between TM3 and TM6. Class C GPCRs have an ionic lock (Doré et al., 2014) whereas Class B GPCRs do not (Hollenstein et al., 2013). Recent structural data from FZD_4 and homology models based on SMO point to the existence of a traditional GPCR-like binding pocket (orthosteric in Class A/B; allosteric in Class C) in the transmembrane domain – the accessibility of which has been brought into question (Yang et al., 2018).

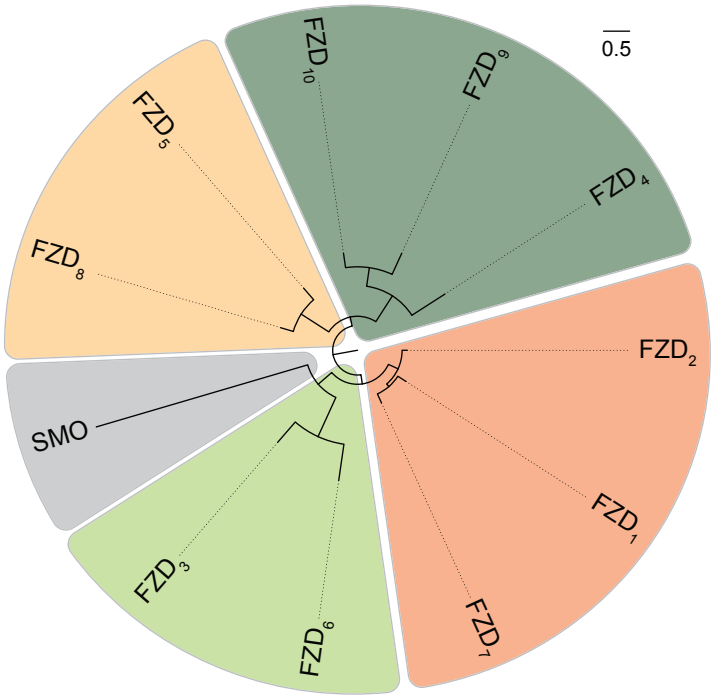

Fig. 2. Phylogenetic tree of human FZD and SMO paralogues. Maximum-likelihood phylogenetic tree depicting the homology clusters of all Class F members.

In short, the differences and similarities between Class F (**Fig. 2**) and the rest of the GPCR superfamily underline the evolutionary trajectories that allowed for adaptation to different ligands in different cellular/tissue contexts that converge on related intracellular transducers yielding different responses of varying amplitudes and kinetics.

1.3.2 Ternary complex model and GEF activity

The observation that agonist/hormone (*H*) affinity for the receptor (*R*) was sensitive to nucleotides led to the postulation of a ternary complex model whereby the G protein (*X*) stabilizes the complex in the absence of enzymatic activity where K, L, K′ and M are constants (De Lean et al., 1980; Limbird et al., 1980). In other words, an agonist will have a higher affinity for the receptor when functionally interacting with the G protein (**Fig. 3**). This observation is reinforced on a structural level where a lid-like structure encapsulates the receptor-bound ligand when the receptor is functionally engaging the intracellular transducer (Devree et al., 2016). These findings were extended to other 7TM receptors as well as other intracellular effectors – both physiological and surrogate-based. Physiological ternary complexes for which the affinity shift of an agonist-bound receptor has been observed include heterotrimeric G protein, arrestin (Gurevich et al., 1997) and G protein-coupled receptor kinase (GRK) (Komolov et al., 2017) (**Fig. 4**). G protein surrogates in the form of nanobodies or engineered G protein can also induce the affinity shift characteristic of a high affinity complex and have provided structural insight into ternary complex formation where sufficient stabilization of protein structure has been problematic (Manglik et al., 2017; Shimada et al., 2018). Findings that GPCRs can preassociate with G proteins in the absence of ligand and that

allosteric modulators can affect agonist affinity led to the development of the cubic ternary complex and the quaternary complex model respectively (Christopoulos and Kenakin, 2002; Weiss et al., 1996) – the inclusion of more variables will inevitably lead to more complicated models.

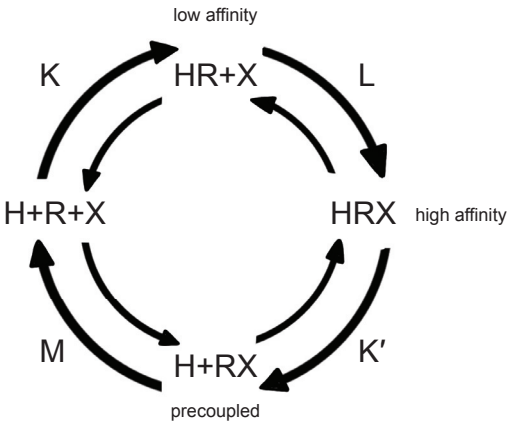

Fig. 3. Ternary complex model. Observations that an agonist had different affinities for its receptor depending on the presence or absence of intracellular transducer led to the development of the ternary complex model. – Adapted from De Lean et al., 1980 and illustrated by Jennifer M. Wright

Heterotrimeric G proteins are composed of three subunits: Gα, Gβ and Gγ. In particular, the Gα subunit acts as a weak GTPase in the absence of regulators of G protein signalling (RGS) by catalyzing the hydrolysis of GTP (active) to GDP (inactive) and consists of two domains that are involved in nucleotide binding: the Ras domain and the α-helical domain. Like GPCRs, Gα subunits are divided into different families: $G\alpha_s$, $G\alpha_{i/o}$, $G\alpha_{q/11}$, $G\alpha_{12/13}$ (Milligan and Kostenis, 2006). It is well-established that GPCRs exhibit a degree of G protein specificity and that some GPCRs can interact with more than one subfamily of G protein through selectivity determinants (Flock et al., 2017). This selectivity is achieved through contacts between the receptor and the Ras domain of the Gα subunit. The α-helical domain of the Gα subunit is flexible allowing for exchange of GDP for GTP (Rasmussen et al., 2011a). The exchange of GDP for GTP is facilitated by the guanine nucleotide exchange factor (GEF) activity of the GPCR. The outward movement of TM6 on the receptor exposes a larger surface area for the α5 helix of the Ras

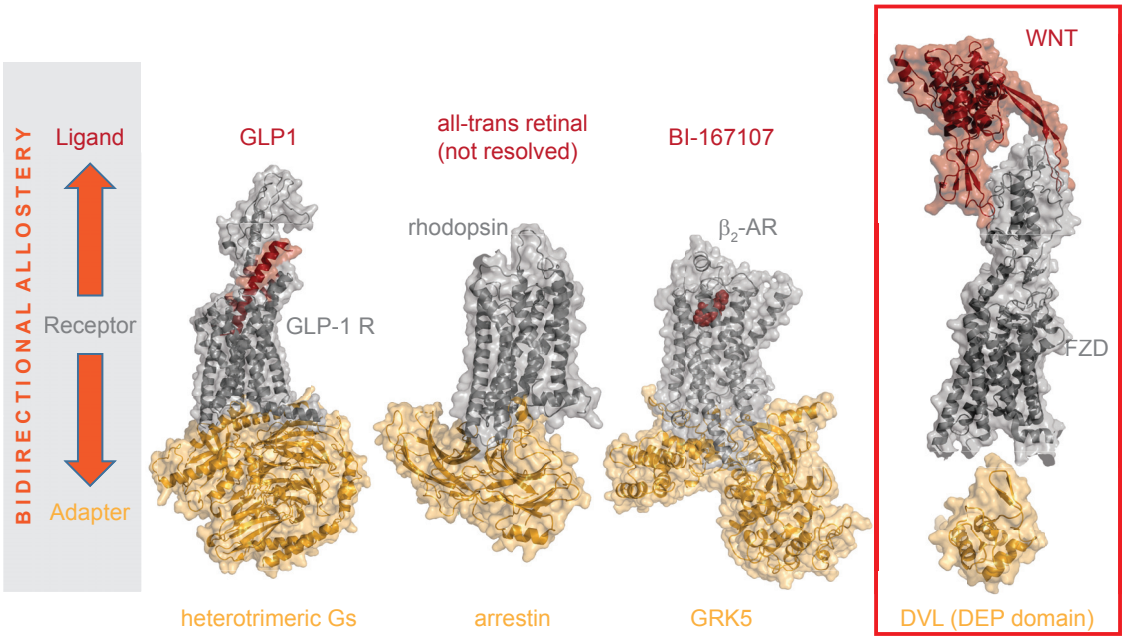

Fig. 4. Ternary receptor complexes. The term GPCR has become a misnomer due to the ability of other intracellular effector proteins to bind the receptor and allosterically affect agonist affinity. Heterotrimeric G proteins, arrestins and GRKs can all form ternary complexes. With respect to Class F receptors, the question arises whether other proteins like DVL for FZDs or Cos2 for SMO in *Drosophila* can also form ternary complexes. – Adapted from Schulte and Wright, 2018

domain to become fully engaged with the cytoplasmic cavity of the 7TM bundle (Carpenter and Tate, 2017). This results in the opening of the nucleotide binding site and subsequent displacement of GDP. The nucleotide-free state of the G protein is short-lived due to the relatively high levels of GTP present in the cell. Then, either the heterotrimer undergoes a structural rearrangement or Gβγ dissociates from Gα where they can act as effectors elsewhere in the cell (Bunemann et al., 2003; Digby et al., 2006; Galés et al., 2006). Previously, the life cycle of a GPCR was thought to be complete after dissociation of the heterotrimeric G protein from the agonist-bound receptor, which leads to phosphorylation by GRKs and receptor desensitization by arrestins. However, it is now understood that GRKs bind to the agonist-bound receptor – albeit with a weaker affinity than the heterotrimeric G protein – to form an alternative ternary complex. Phosphorylation of the receptor by GRK leads to the recruitment of arrestin which results in receptor internalization and rheostat-like signalling that is modulatory of G protein signalling (Grundmann et al., 2018; Irannejad et al., 2013; Luttrell et al., 2018).

In the context of FZDs, selected paralogues have been found to assemble with heterotrimeric G proteins in an inactive state (although the presence of endogenous WNTs could be a confounding factor) and DVL, which can interact with FZD in a ligand-independent manner (Arthofer et al., 2016; Hot et al., 2017; Kilander et al., 2014; Valnohova et al., 2018). It has also been shown that LRP5/6 can directly bind to FZD (Ren et al., 2015) – a finding that requires further investigation to determine how this interaction affects WNT-FZD-transducer cooperativity.

1.3.3 Oligomerization

Dating back to the 1970s, bindings curves that revealed differences in cooperativity were proposed to support the existence of higher order complexes with more than one binding site (Bouvier, 2001). Findings from co-immunoprecipitation experiments and immunoblotting have provided evidence for dimers/higher order oligomers (**Fig. 5A**). This approach was used to demonstrate that *Xenopus* FZD_3 dimerizes whereas FZD_7 does not. It was further shown that by artificially constraining FZD_7 to form dimers, the WNT/β-catenin pathway could be activated (Carron et al., 2003). However, receptor solubilization can induce artefacts due to the hydrophobic nature of GPCRs and domain swapping does not provide information about the native protein function. In order to circumvent this, there was a push in assay development to probe for the presence of dimers in living cells. Bioluminescence resonance energy transfer (BRET) is a process that occurs naturally in the sea pansy, *Renilla reniformis*. Degradation of coelenterazine by luciferase results in luminescence that can be transferred to green fluorescent protein (GFP) when the two proteins are in proximity to one another (<100 Å). Through molecular cloning, luciferase and red-shifted GFP can be fused to proteins of interest. In the present context, two populations of receptors can be tagged with donor and acceptor molecules enabling the assessment of dimers in living cells (Angers et al., 2000) (**Fig. 5B**). BRET assays suggested that $FZD_{1,2,4}$ dimerize via their heptahelical domain and that this process is important in receptor trafficking (Kaykas et al., 2004). However, the inability to distinguish random

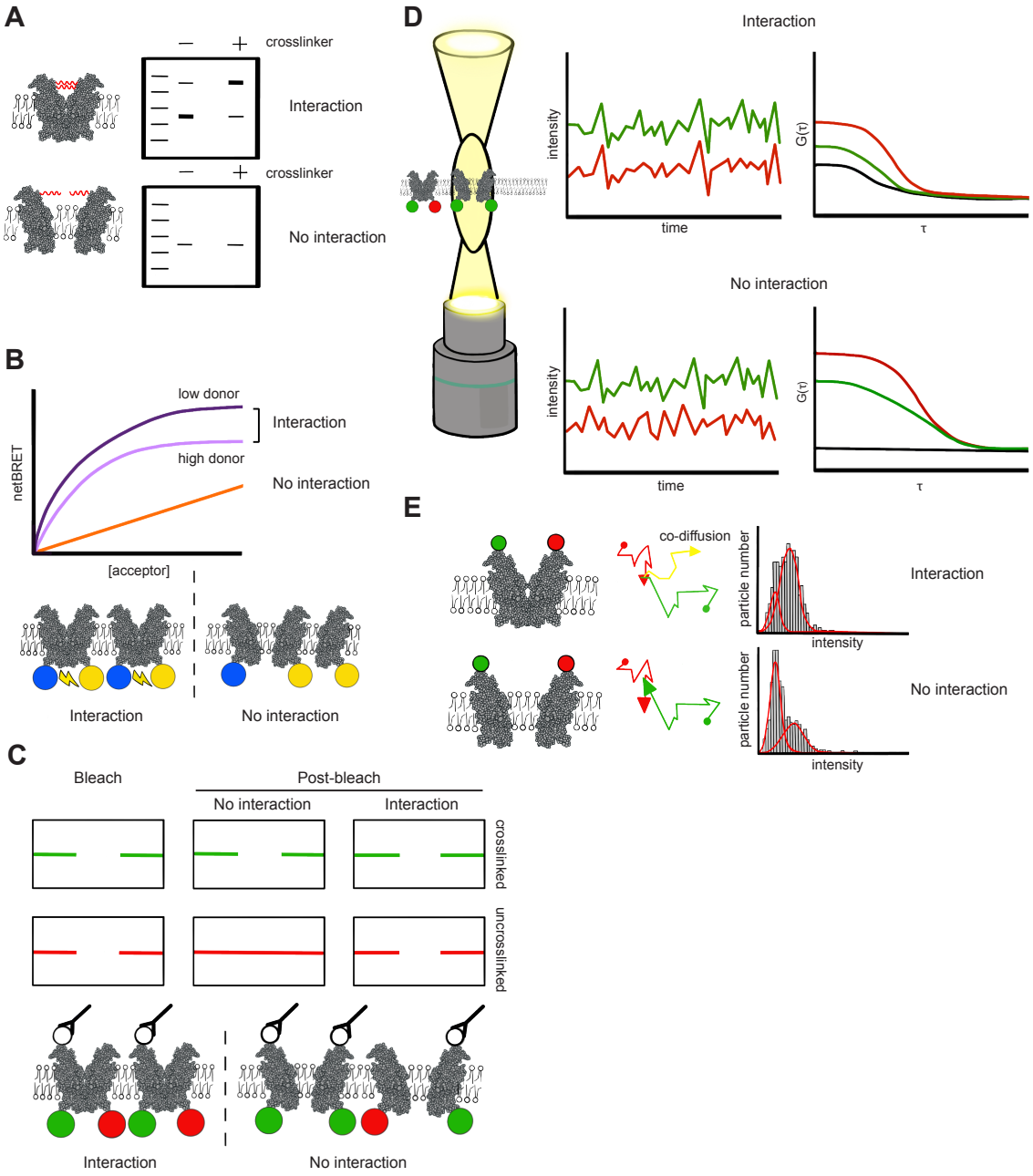

Fig. 5. Methods to detect dimerization. Over the last decades, methods have developed or been adapted to increase sensitivity, account for stoichiometry and quantitatively assess oligomerization. (**A**) Immunoblotting has been used to probe for dimers based on their relative size with the help of antibodies in co-immunoprecipitation experiments or crosslinkers of varying lengths and chemical reactivity. (**B**) BRET is performed in living cells and provides an indication of the relative distance between proteins based on energy transfer from luciferase-tagged proteins to red-shifted GFP-tagged proteins. In order to convincingly show that RET is dependent on stoichiometry and not just acceptor density, different amounts of donor DNA should be transfected. Typically, the expression of acceptor should exceed expression of the donor. (**C**) FRAP experiments employing two colours can be used to measure the interaction between two protein populations when one population represented by a particular colour (in this case green) can be immobilized by antibody or crosslinker. The two colours (protein populations) are bleached and the recovery of the mobile protein population (red) is monitored for differences in its lateral mobility before and after immobilization of the green population. (**D**) FCCS tracks the movement of fluorescently-tagged molecules through a detector volume over time. If two proteins are interacting or co-diffusing then a cross-correlation amplitude will be observed. (**E**) Single-molecule tracking of proteins labelled with cell-impermeable fluorescent dyes has been used to acquire information about the size of particles over varying receptor densities and co-diffusion of molecules. – Illustration by Jennifer M. Wright

collisions from stable interactions and problems in estimating stoichiometry with BRET led to the use of saturation curves that have become the subject of debate and controversy in the field of GPCR dimerization (Lan et al., 2015) (see **3.3 Affinity vs. proximity and the role of stoichiometry**). Another approach known as dual color fluorescence recovery after photobleaching (dcFRAP) made use of fluorescent receptors where one receptor population could be immobilized by antibodies or crosslinkers in order to test whether the other receptor population would be slowed in its lateral mobility (Digby et al., 2006; Dorsch et al., 2009) (**Fig. 5C**). Problems inherent to receptor overexpression and the need for receptor crosslinking led to the use of assays capable of tracking single molecules over time. Fluorescence correlation spectroscopy (FCS) has single molecule sensitivity and can measure fluorescence fluctuations within a defined detector volume providing information about movement, concentration and aggregation (**Fig. 5D**). When protein populations labelled with two colours are expressed in living cells, their movement can be correlated to determine whether the proteins are in complex or not. However, this method is technically difficult to carry out, suffers from photobleaching if red-shifted fluorescent protein chimeras are used and does not rule out the presence of other protein intermediates that allow for correlated movement (Briddon et al., 2018). With the help of algorithms, another technique with single molecule resolution made use of total internal reflection fluorescence microscopy (TIRF-M) to study receptor organization in living cells (**Fig. 5E**). These studies highlighted the dynamic nature of receptor-receptor interactions that were highly variable in their sensitivity to ligand addition (Calebiro et al., 2013). In spite of these findings, the functional relevance of dimers was still an open question. The discovery that purified GPCRs could be embedded into high-density lipoprotein (HDL) particles as monomers provided strong evidence for the ability of a monomer to activate G proteins in the presence of receptor agonist. Furthermore, the authors proposed that the difference in cooperativity was due to G protein binding and not dimerization (Whorton et al., 2007). While the aforementioned study yielded direct support to the ternary complex model (De Lean et al., 1980), it does not preclude the existence of dimers or the potential that they may have some effect on G protein activation. For example, members of the Class C form covalent dimers via their ligand-binding domain and this has functional consequences for receptor activation (Xue et al., 2015). To sum up, the difficulties in measuring stoichiometry, defining oligomeric interfaces and excluding protein intermediates have slowed down our pharmacological understanding of the relevance of GPCR dimers, which necessitates further studies.

1.4 CLASS F PHARMACOLOGY AND CONFORMATIONAL BIAS

A great deal of confusion revolves around the pharmacology of Class F receptors. Instead of finding common ground on a structural level given their sequence homology, the peculiarities of SMO compared to other Class F members has driven research efforts in opposing directions.

SMO was long thought to be a constitutively active orphan receptor, activated indirectly by Hh and repressed through an unknown mechanism by PTCH. A curious observation that ingestion of a particular plant species by pregnant sheep could induce cyclopia in fetal lambs was ultimately shown to involve the binding of a teratogen called cyclopamine to the 7TM bundle

of SMO (Chen et al., 2002; Keeler and Binns, 1966). Given the importance of oncogenic SMO in cancer, the finding that the receptor core could be modulated by small molecules provided a logical starting point for drug development. In fact, while drug development for SMO has focused on its GPCR architecture, there is still a debate as to whether it functions in a physiological context as a GPCR (Arensdorf et al., 2016). The first structure of a Class F member was that of human SMO bound to the antitumour agent LY2940680 (Wang et al., 2013). In contrast to FZDs, less focus has been placed on the role of the CRD in SMO activity – especially in the design of small molecule drugs – although oxysterols have been found to bind the hydrophobic groove in the CRD and play a role in pathway activation (Byrne et al., 2016). Nevertheless, the rearrangements observed in the binding pocket of agonist- and antagonist-bound SMO structures do not support the differences observed in receptor activity (e.g. SMO-induced Gli transcriptional regulation) (Zhang et al., 2018). Furthermore, the identity of the endogenous SMO ligand is still controversial with some indications that it could be cholesterol or oxysterols (Byrne et al., 2016; Huang et al., 2016; Luchetti et al., 2016; Myers et al., 2013). This problem is complicated by the fact that the endogenous regulator of SMO activity, Hh, binds PTCH and not SMO. The question arises whether SMO has evolved a constitutive activity that is agonist-independent *in vivo*, but repressed in a graded fashion by PTCH under normal circumstances. To date, SMO has not been crystallized with any intracellular transducer. Therefore, we can only speculate about conformational bias and the development of biased ligands. SMO has been shown to interact directly with Costal-2 (Cos2) in *Drosophila*; however, human SMO does not bind Cos2 (De Rivoyre et al., 2006). In addition, G protein-coupled receptor kinase 2 (GRK2), protein kinase A (PKA) and casein kinase 1 (CK1) are known to phosphorylate SMO, but the mechanism through which Hh stimulation and PTCH inactivation allows these kinases to activate SMO is unknown (Chen et al., 2004b; Zhao et al., 2007). However, there is an abundance of literature that demonstrates the ability of SMO to couple to and activate heterotrimeric G proteins (Arensdorf et al., 2017; Myers et al., 2017; Riobo et al., 2006). Both agonists and inverse agonists that target the 7TM bundle of SMO are known to affect G protein activation. For this reason, it would be interesting to uncover the conformational changes and structural motifs involved in receptor activation and GEF activity. Moreover, an understanding of how SMO is conformationally repressed by PTCH would be of enormous value as this remains a big question mark in the field of Hh signalling. The concept of pathway selectivity does not appear to apply to SMO – at least not in the same way that it does to WNT/FZD signalling (see below). However, phosphorylation of SMO leads to the recruitment of β-arrestin – a process that can be inhibited by small molecule inverse agonist (Chen et al., 2004b). As for other GPCRs, one could think of developing biased SMO ligands for the purpose of targeting specific intracellular transducers that are activated by SMO (Hauser et al., 2017).

With regard to FZDs, emphasis has been placed on the CRD in large part due to the WNT-CRD structure and the inexistence of a ligand-bound 7TM structure. This has led to the development of CRD-targeting antagonists – some of which have proceeded to clinical trials. Unfortunately, insufficient paralogue selectivity has resulted in serious issues in

polypharmacology (Schulte and Wright, 2018). Moreover, it is not clear in view of the full-length receptor whether WNT binding to FZD results in a reorientation of the CRD relative to the core resulting in a secondary binding site that contacts residues elsewhere on the receptor to mediate receptor activation. This information is especially important given the evidence that the CRD does not appear to be required for WNT signal transduction through FZD (Chen et al., 2004a). Without a better understanding of the binding mode of WNT proteins, we cannot pinpoint the location of the orthosteric site on full-length FZD. This problem has been compounded by an endemic nomenclature problem in the WNT field that treats proteins and drugs that negatively affect WNT/β-catenin signalling as antagonists. A pharmacological overhaul is needed to recharacterize current treatment modalities and to characterize new ones – all of which requires more structural data (Neubig et al., 2003). As mentioned before, understanding pathway selectivity and bias is far more complex in WNT signalling. Endogenous allosteric modulators of FZDs in the form of co-receptors [e.g. LRP5/6, ROR1/2, RYK, Tyrosine-protein kinase-like 7 (PTK7)] or dimers could affect cooperativity of ligand binding according to the allosteric ternary complex model, dictate specificity among the 19 WNTs and influence pathway selectivity (Christopoulos and Kenakin, 2002; Eubelen et al., 2018; Liang et al., 2018a; May et al., 2007). The first structure of the 7TM domain of FZD – that of FZD_4 – revealed a hydrophilic pocket like SMO that was claimed to be unfavourable for ligand binding (Yang et al., 2018). Interestingly, the first crystal structure of SMO in complex with LY2940680 made similar claims about the size and hydrophilicity of the pocket (Wang et al., 2013). The fact that the FZD_4 structure is in the apo state – the first such 7TM structure – suggests that the narrow binding pocket could be partially collapsed. It is attractive to speculate that FZDs evolved Class F-specific conformational switches that take part in GEF activity and dynamic kinks in TM7 that mediate interaction with other binding partners like DVL (Yang et al., 2018). Further studies into the conformational changes that guide pathway selectivity will drive drug discovery efforts forward.

1.5 SIGNALLING KINETICS AND SIGNAL AMPLIFICATION IN WNT/HH SIGNALLING

The long held belief that the response induced by a drug is proportional to the number of occupied receptors was brought into question after it was shown that the histamine receptor antagonist phenoxybenzamine was resistant to washout, but stimulation of unbound receptor reserve with histamine could still elicit a maximum response until all receptors were occupied by antagonist (Nickerson, 1956). As mentioned previously, the receptor-mediated GEF activity is sufficiently rapid such that one receptor can activate many G proteins – one molecule of rhodopsin can activate as many as 1000 molecules of transducin during one activation/deactivation cycle (Vuong et al., 1984). The potential of the receptor to amplify the signal through the subsequent activation of many effector molecules implies that lower concentrations of agonist can result in full activation of downstream responses (Ross, 1989). These observations were instrumental in developing concepts of spare receptors/receptor reserve and signal amplification. Understanding that these factors may be at play when studying receptor function is essential when choosing the appropriate assay and concentration

of ligand. In order to tie WNT and Hh signals to cellular signalling events, gene reporter assays were developed to measure the transcription of target genes (Korinek et al., 1997; Sasaki et al., 1997). These luciferase-based reporter assays were based on the co-activator β-catenin binding to the transcription factor TCF to bind the *c-Fos* promoter or the transcription factor Gli binding to Gli responsive genes like the enhancer for hepatocyte nuclear factor-3β (*HNF-3β*) for WNT and Hh signalling respectively. The time that it takes for luciferase to be expressed after ligand stimulation is considerably longer than the time it takes to measure the responses that take place upstream. Taking receptor activation as an example, conformational changes are quick and transient requiring higher concentrations of ligand than downstream readouts to achieve the maximum response. Using recombinant WNT or Hh proteins at saturating concentrations will activate effectors at all levels of the signalling cascade; however, the concentration of ligand contained in conditioned medium may be insufficient to detect responses upstream, but largely sufficient to measure downstream, end point readouts (including the caveat that quantification of agonist concentration in conditioned medium is intrinsically cumbersome). Given the ubiquitous expression of Class F receptors and redundancy among paralogues, the end point responses are more than likely a combination of receptor reserve and signal amplification. Building on this idea, it would be interesting to investigate how different concentrations of ligand and redundancy among FZD paralogues feeds into the channel capacity of systems dependent on WNT signalling (Keshelava et al., 2018). In view of this, ignoring the signalling events that take place immediately following ligand-receptor binding and the catalytic potential for proteins to amplify signals over several orders of magnitude is worrisome for the understanding of upstream signalling mechanisms and drug discovery. In the case of WNT signalling, there are certainly some open questions regarding the kinetic discord between WNT/β-catenin signalling and β-catenin-independent signalling. Are these pathways truly independent of one another or is there a justifiable kinetic component that integrates this all together (i.e. negative feedback loop or crosstalk)?

2 SPECIFIC AIMS

The main objective of this thesis is to understand the mechanisms underlying Class F receptor activation. The specific aims are to:

- Gain insight into FZD complex stoichiometry and dynamics
- Study the GPCR nature of FZDs in the context of pharmacology
- Define dynamic conformational changes involved in Class F activation
- Aim to integrate findings between FZDs and heterotrimeric G proteins into cell models with physiological and pathophysiological relevance
- Understand structural changes underlying agonist-induced or constitutive receptor activation
- Develop assays that can be used to screen for FZD-targeting small molecules

3 MATERIALS AND METHODS

All materials and methods that have been used to produce the results that make up this thesis (see **Table 1**) are described at length in the papers themselves. However, there are some methodological considerations that will be discussed herein.

3.1 DECIPHERING WNT-FZD SELECTIVITY

Table 1. Methods used to produce the data presented in this thesis.

Method	Paper
AlphaScreen	III
BRET	II, III
Ca^{2+} mobilization	II, III
CD	I
Cell culture	I, II, III
Cell viability assay	II
Confocal microscopy	I, II, III
CRISPR/Cas9	I
dcFRAP	I, II
DMR	II
FC(C)S	I
FRET	II
Immunoblotting	I, II, III
ITC	I
Molecular modeling	I, II, III
Mutagenesis	I, III
SEC	I
Transfection	I, II, III

WNTs are ubiquitously expressed across cell types and there is no concrete method for quantifying the amount of protein expression of the individual paralogues. Not surprisingly, FZDs are also expressed in a ubiquitous manner and it is known that a certain degree of redundancy exists amongst them (Bhanot et al., 1999; Wang et al., 2006). This has slowed down efforts to nail down WNT-FZD selectivity and has pushed for the development of a number of tools in recent years. Initial efforts focused on finding cell types that expressed select FZDs or little to no FZDs at the mRNA level. This led to the use of mouse bone marrow derived 32D cells that expressed little to no FZD mRNA and were unresponsive in classical WNT signalling readouts without the introduction of exogenous FZD (Dijksterhuis et al., 2015). However, these cells were difficult to transfect and only a few stable lines could be generated. A more protein-centric approach utilized isolated anti-FZD Fabs that collectively became known as the "FZD profiler" – a flow cytometry-based method to discriminate the expression of FZD paralogues (Steinhart et al., 2017). With this knowledge in hand, one can attribute signalling responses to particular paralogues with greater certainty. During the same time, efforts were ongoing to multiplex CRISPR/Cas9-mediated gene editing in order to knockout all FZD paralogues. This resulted in two mutant HEK293 cell lines: $FZD_{1,2,4,5,7,8}^{-/-}$ (Voloshanenko et al., 2017) and $FZD_{1\text{-}10}^{-/-}$ (Eubelen et al., 2018) – the latter being completely depleted of FZDs. So far, no group has succeeded in knocking out all 19 WNT paralogues. Packaging cell lines like HEK293 cells have been the first choice for gene inactivation experiments due to their readiness to accept foreign DNA. In cell types that have a normal karyotype, the essentiality of the gene in question can result in a loss in viability.

For **Paper I**, a number of CRISPR-based approaches were also used to study the role of endogenous FZD_6 in MLE-12 cells. Firstly, wild-type Cas9 was directed to exon 3 of *FZD6*

and clonal analysis was performed to screen for homozygous knockouts. For reasons unknown, only heterozygous lines were generated that had reduced FZD_6 expression and were deficient in phosphorylation of ERK1/2 and their ability to proliferate. Another approach made use of the endonuclease-incapable dCas9 that was directed to the transcription start site (TSS) of *FZD_6*. Although differences could be observed at the mRNA level, protein expression was not consistently different – possibly due to tight regulation of mRNA translation irrespective of CRISPR interference (unpublished data). The opposite effect was seen after CRISPR activation using dCas9-VP160 directed to the promoter of *FZD_6*. An increase in mRNA levels of *FZD_6* led to greater protein production resulting in more phosphorylation of ERK1/2.

Because of the focus on FZD_6 in **Paper I**, the physiological relevance of using a cell type that depends on FZD_6 expression seemed logical. In retrospect, it may have been easier to use some of the aforementioned tools – had they existed at the time – to 1) confirm protein expression of all paralogues in MLE-12 cells or 2) use a packaging cell line depleted of all FZD paralogues and reintroduce FZD_6, but with less physiological relevance. Moreover, the effects mediated by FZD_6 in MLE-12 cells were not controlled for WNT-induced activation and therefore could be the result of constitutive activity. See **3.2 Inverse agonism, constitutive activity and method sensitivity** for more information.

3.2 INVERSE AGONISM, CONSTITUTIVE ACTIVITY AND METHOD SENSITIVITY

The discovery of small molecules that target WNT secretion by inhibiting Porcupine (Liu et al., 2013; Proffitt et al., 2013) – the protein that acylates WNTs – enables one to create a biological system that is free of WNTs and this allows for measurements of constitutive activity in the presence of overexpressed FZD. Because inverse agonists that bind to FZDs have not yet been discovered, measuring the constitutive activity of endogenous FZDs is not currently possible.

Nevertheless, small molecule inhibitors that prevent WNT secretion can allow us to study FZD function in an overexpression paradigm with the caveat that the ability to say with absolute certainty whether a receptor displays constitutive activity depends on the sensitivity of the method. A prime example of this is apparent from the results presented in **Paper I** and **Paper III**. In **Paper I**, it was concluded from semi-quantitative immunoblotting that in the absence of endogenous WNTs, overexpressed wild-type FZD_6 did not display constitutive activity. In **Paper III**, the more sensitive and quantitative AlphaScreen assay was also used to measure constitutive activity of wild-type FZD_6 – this time in comparison to another mutant – and despite there not being sufficient statistical power, there was a visible difference in the amount of activity compared to the control condition. In short, as the methodologies become more sensitive, we will gain greater insight into the action of drugs or the activity of proteins that were not previously measurable.

3.3 AFFINITY VS. PROXIMITY AND THE ROLE OF STOICHIOMETRY

Receptor complex formation is an important area of research in WNT signalling where numerous co-receptors, scaffolding proteins and intracellular effectors have been implicated in a plethora of signalling pathways (Schulte and Wright, 2018). In order to piece together the building blocks that create functional receptor complexes, an understanding of protein complex stoichiometry is required – that is to say the relative amount of one protein to another.

In **Paper I** and **Paper II**, dcFRAP was used to measure receptor-receptor interaction and receptor-effector interaction in combination with antibody-mediated and chemical crosslinking respectively. In both instances, an excess of the crosslinked protein was needed to ensure that if there was an interaction, then it would be detectable. To this end, an artificial transmembrane protein consisting of the two fluorophores used in the assay (mCherry and GFP) was constructed. This tool was aptly called a stoichiometer because it allowed for an approximation of the relative amount of one protein to the other (Petersen et al., 2017). Equating the relative fluorescence intensity of one fluorophore to the other ensured that dcFRAP experiments were comparable and strengthened our findings. Since dcFRAP experiments rely on the diffusion of unbleached, uncrosslinked proteins into an area where crosslinked proteins have been immobilized, we can conclude that if the uncrosslinked proteins are restricted in their movement, this is due to an affinity for the crosslinked proteins. By consequence, shifting stoichiometry in favour of the crosslinked population should result in a greater immobilization of the uncrosslinked population if there is indeed an interaction. The inclusion of appropriate positive or negative controls such as monomeric or dimeric reference proteins, membrane-tethered fluorescent proteins or artificial single-pass transmembrane proteins fused with fluorescent proteins should support the data (Arthofer et al., 2016; Digby et al., 2006; Dorsch et al., 2009).

Contrary to dcFRAP, RET-based approaches provide an indication about the proximity between two proteins (10 nm or 100 Å). Without proper controls and/or complementary techniques, conclusions about protein interaction cannot be determined by RET. Building on concepts derived from radioligand binding assays assuming 1:1 stoichiometry, the idea to titrate acceptor concentration in BRET experiments in an attempt to perform saturation analysis has led to conflicting reports regarding the absence or presence of a given interaction (e.g. receptor dimerization) (Lan et al., 2015; Mercier et al., 2002). Early seminal studies demonstrated that the amount of energy transfer between two interacting proteins depended on the stoichiometry between donor and acceptor rather than simply acceptor expression. In other words, energy transfer should increase irrespective of whether two proteins specifically interact with one another or collide randomly with one another; however, the nature of this increase will be linear rather than hyperbolic (Kenworthy and Edidin, 1998; Lan et al., 2015; Wan et al., 2018; Wolber and Hudson, 1979). If energy transfer is to be deemed specific, then it must also depend on donor concentration. It has become standard practice when performing saturation analysis in BRET experiments to plot energy transfer as a function of acceptor to donor. While the underlying assumption is that the amount of donor DNA has been kept constant, cellular machinery can favour expression of the acceptor at higher concentrations,

thereby reducing expression of the donor. This can transform data that would normally be linear into hyperbolic. Although this type of data transformation facilitates the pooling experimental replicates, it neglects the fact that protein expression does not always mirror DNA transfection. For that reason, controls should be carried out to confirm the presence or absence of a presupposed interaction (i.e. two known non-interacting proteins, mutated binding site/interface). RET can also be used to harness the random, non-specific interactions. This type of RET – known as bystander RET – relies on subcellular markers to ascertain where a protein of interest is localized (Lan et al., 2012).

3.4 SPECIFIC ACTIVITY AND FZD PHARMACOLOGY

Current methodologies allow for two main ways to stimulate cells/organoids with WNTs: conditioned medium and recombinant protein. WNTs are post-translationally modified through acylation and lipidation affecting trafficking and solubility (Janda et al., 2012; Langton et al., 2016). While the WNT-conditioned medium produced from L-cells is cheaper and more stable under culture conditions, it is not pure nor do we have any idea what other co-factors or proteins may confound the response being measured. Therefore, it is not possible to perform accurate concentration-response experiments. Moreover, the concentration of WNT present in conditioned medium is much lower than commercially-available, purified, recombinant WNT. This is not a problem when measuring signal-amplified downstream readouts, but becomes an issue when higher concentrations are required to measure protein activation at the level of the receptor complex. Whereas conditioned-medium certainly has its place in many functional readouts (e.g. transcriptional readouts) as well as in the maintenance of stem cell/organoid cultures, it is not used in studies of FZD pharmacology. Protein biochemists and pharmacologists alike have struggled in purifying the WNT paralogues due to their high lipophilicity and susceptibility to protein degradation/inactivation. Nowadays, several WNTs are commercially available with or without carrier protein (e.g. bovine serum albumin). One of the drawbacks to using recombinant WNT is the variable specific activity between WNT paralogues.

Based on previous experiments carried out on serum-deprived microglia cells and the sensitive nature of mitogen-activated protein kinase (MAPK) responses, WNTs without carrier were used in **Paper I** to measure the phosphorylation of ERK1/2. In contrast to this, information was collected from several recent publications (Dhamdhere et al., 2014; Mihara et al., 2016; Tüysüz et al., 2017) and in discussion with other GPCR pharmacologists, several measures were taken to produce the concentration-response curves in **Paper II** and **III**. Namely, recombinant WNT with carrier was maintained in assay buffer containing equimolar carrier protein. Serial dilutions of ligand were carried out in coated plastics to minimize protein adsorption. Where possible, experiments were performed using freshly reconstituted WNT that was maintained on ice until stimulation at room temperature. These methodological considerations ensured the reproducibility of these findings. More work is required to circumvent this problem in working with the endogenous FZD ligand or a switch must be made to small molecules that are easier to handle, less costly and more robust.

4 RESULTS AND DISCUSSION

4.1 AGONIST-INDUCED DYNAMICS OF FZD DIMERS AND SIGNALLING

The observation that DVL had a modulatory role on heterotrimeric G protein coupling to FZD_6 led us to hypothesize that a higher order complex was required to accommodate the binding of both DVL and G protein (Kilander et al., 2014). This hypothesis was consistent with the finding that other Class F members dimerize (Kaykas et al., 2004; Wang et al., 2013) and the steric requirement for DVL to contact ICL1-3, the H8 KTxxxW motif and the lipid headgroups (Gammons et al., 2016; Wong et al., 2000). The intracellular surface area required to accommodate both DVL and G protein does not appear to follow the same mechanism described by the recent discovery of a monomeric GPCR that can engage both G protein and β-arrestin simultaneously (Thomsen et al., 2016).

In **Paper I**, we pursued this rationale and demonstrated via dcFRAP and FCCS that FZD_6 forms dimers – data that were supported by a SMO crystal structure (Wang et al., 2013). Mutational analysis and expression of dimer-interfering minigenes linked the dimer interface to TM4/5. Upon receptor activation, the α-helical domain of the Gα subunit of the G protein is predicted to undergo a conformational switch that would sterically clash with a TM4/5 dimer (Cordomí et al., 2015). This was confirmed by dcFRAP and FCCS after stimulation with the FZD ligand WNT-5A, which led to the transient dissociation of FZD_6 dimers. In order to study the physiological signalling output of FZD_6, we turned to cells derived from the distal bronchiolar and alveolar epithelium of mouse lung epithelium – MLE-12 cells. The addition of recombinant WNT-5A to MLE-12 cells resulted in a time-dependent increase in ERK1/2 phosphorylation that kinetically coincided with the dissociation/reassociation of FZD_6 dimers. CRISPR/Cas9-mediated activation and inactivation of FZD_6 in MLE-12 cells showed that ERK1/2 phosphorylation is regulated by FZD_6 expression in the presence of endogenous WNTs. Given the G protein-mediated activation of ERK1/2, these findings merged well with previous data demonstrating that FZD_6 was a Gi/q-coupled receptor (Kilander et al., 2014). In HEK293 cells treated with Porcupine inhibitor, expression of the FZD_6 dimer mutant continued to signal to ERK1/2 in a ligand-independent manner contrary to wild-type FZD_6.

Further investigation is required to understand how the FZD_6 dimer mutant is capable of signalling to ERK1/2 while not being able to preassociate with $G\alpha_{i1}$. One possibility is that FZD_6 monomers are incapable of forming stable complexes with heterotrimeric G proteins and interact via collision coupling. Further experiments are required to confirm this hypothesis, but this could be tested in a RET-based set-up using donor-tagged Gα and acceptor-tagged Gβγ (Andressen et al., 2018). In sum, this suggests that while FZD_6 monomers are sufficient to initiate signalling, dimers represent the inactive, ligand-responsive form of FZD_6 as summarized in **Fig. 6**. These findings are supported by the fact that all active Class A and B GPCR structures to date have been monomeric (Gurevich and Gurevich, 2018).

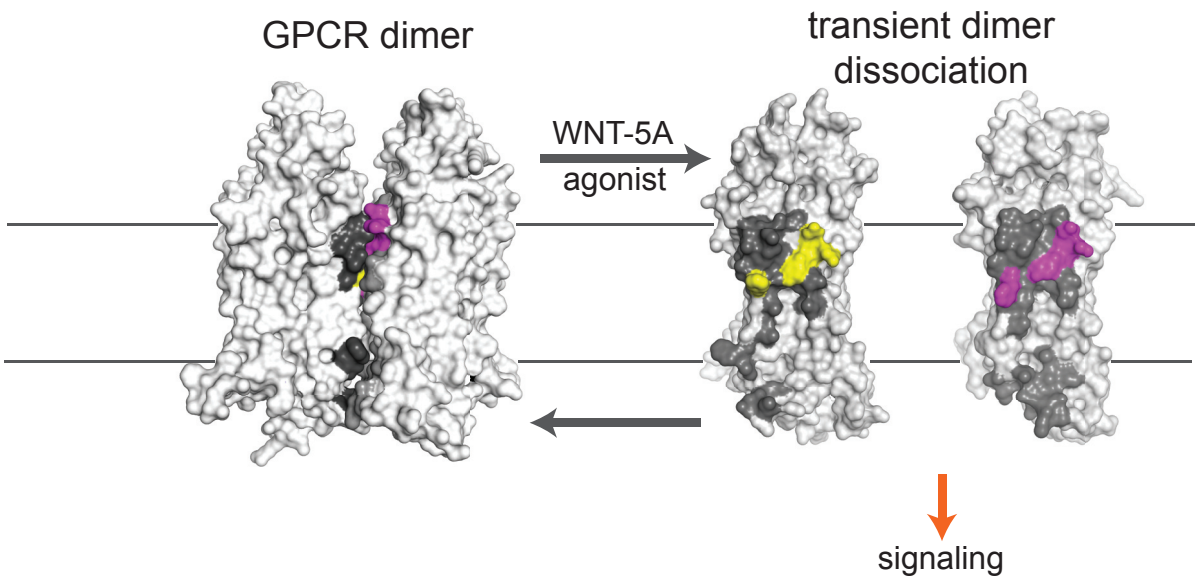

Fig. 6. Agonist-dependent dynamics of FZD_6 dimerization. FZD_6 dimerizes via TM4/5 and after binding of WNT-5A, it dissociates to produce active signalling monomers that will reassociate upon signal termination.

4.2 FZDs UNDERGO CONFORMATIONAL CHANGES CONSISTENT WITH GPCR ACTIVATION

The structural changes that coincide with GPCR activation were largely elucidated by the crystal structure of the fully active β_2 adrenergic receptor in complex with heterotrimeric Gs. Compared to inactive structures, the active structure exhibited a large outward movement of TM6 (Rasmussen et al., 2011a). In recent years, a number of Class A/B GPCR structures have been solved in complex with heterotrimeric G proteins or G protein surrogates (e.g. mini G proteins or nanobodies) (Carpenter et al., 2016; García-Nafría et al., 2018; Liang et al., 2018b; Rasmussen et al., 2011b; Tsai et al., 2018; Zhang et al., 2017). Conflicting structural information and controversy within the Class F has befuddled our understanding of how these receptors transduce signalling. Similar to FZDs, there has also been a resistance to accept SMO as a GPCR due to its strong link to Hedgehog signalling (Arensdorf et al., 2016). Drawing parallels with prototypical GPCRs, it was suggested that cholesterol and cyclopamine bind the CRD to activate SMO by inducing a conformational change in TM6 in the absence of G protein (Huang et al., 2016); although, this could be explained by the insertion of a crystallization scaffold into IL3 (Lefkowitz et al., 2008). Contrary to this, molecular dynamics simulations were run on the inactive structure of ΔCRD-FZD_4 in a ligand-free state and TM6 remained closely packed in the transmembrane domain (Yang et al., 2018). While GPCR structures represent snapshots of a particular conformational state, molecular dynamics simulations provide information about the propensity of a GPCR to sample different conformations and the lifetimes of those conformations (Dror et al., 2011).

These inconsistencies within the Class F necessitate further studies in order to elucidate the structural changes resulting upon ligand binding. In **Paper II**, we used a fluorescein arsenic hairpin binder (FlAsH)-based FRET approach to monitor receptor activation of FZD_5. Based

on knowledge of the outward movement of TM6 upon receptor activation and studies using a similar approach on Class A GPCRs (Hoffmann et al., 2005; Maier-Peuschel et al., 2010; Ziegler et al., 2011), we cloned the FlAsH binding motif into the third intracellular loop of FZD_5 and fused cyan fluorescent protein (CFP) with its C-terminus. The addition of recombinant WNT-5A led to a decrease in FRET between CFP and FlAsH suggestive of a conformational change not unlike prototypical GPCRs (**Fig. 7**). Due to the technical difficulties in radioactively labelling WNTs, this approach represents a major development in assessing WNT-FZD specificity – the results obtained are equivalent to a ligand binding assay. Future studies will certainly exploit this tool to unravel the specific interactions between the 19 WNTs and 10 FZDs with the caveat that their potencies and efficacies cannot be compared without an idea of the specific activity of purified ligands (see **3.4 Specific activity and FZD pharmacology**).

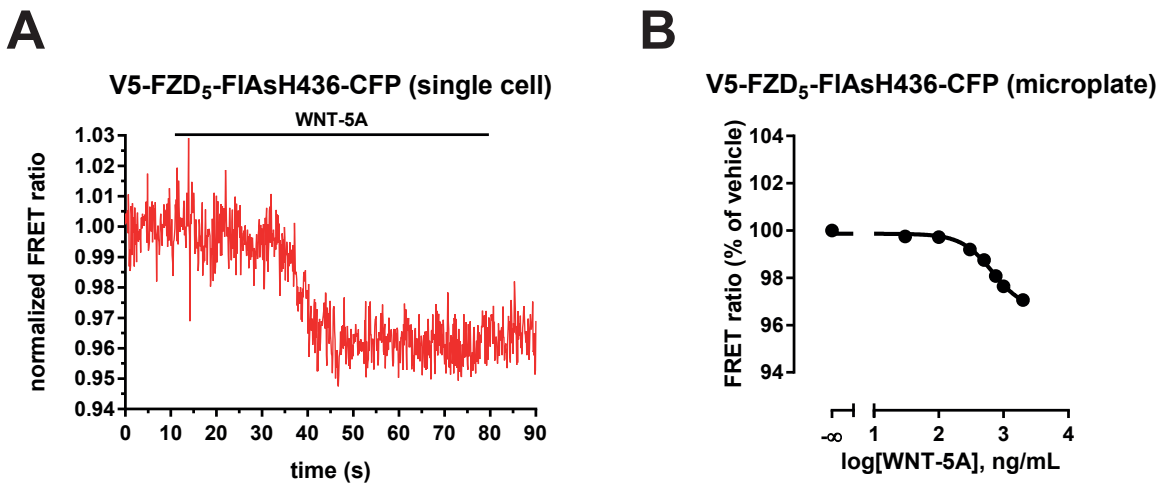

Fig. 7. FZD_5 undergoes a conformational upon agonist addition. (**A**) Stimulation of the FZD_5-FRET sensor with a maximum concentration of recombinant WNT-5A leads to a decrease in RET between the donor-tagged C terminal tail and the acceptor-tagged IL3. (**B**) Concentration-response experiments with the FZD_5-FRET sensor support the idea that we can measure both ligand binding and conformational changes.

4.3 PHARMACOLOGICAL ASSESSMENT OF FZD FUNCTION

FZDs are poorly understood pharmacologically and this is in large part due to the chemical nature of WNTs (see **3.4 Specific activity and FZD pharmacology**) and the lack of small molecule drugs acting on these receptors (Schulte and Wright, 2018). In **Paper II**, we employed RET-based biosensors to monitor receptor activation and map downstream signalling by generating concentration-response relationships. Although these biosensors are highly engineered to maximize the signal-to-noise ratio, they are robust and reliable tools for reading out GPCR activity (Galés et al., 2006; Saulière et al., 2012). The contribution of endogenous G proteins to the observed responses requires some discussion. Using pharmacological inhibitors of $G\alpha_{q/11/14}$ and phenotypic rescue of $G\alpha_{q/11}$KO cells, we demonstrated that FZD_5 signals through $G\alpha_q$ after application of recombinant WNT-5A using BRET sensors that detect endogenous phospholipase C (PLC) and PKC activity as well as whole-cell activation measured by DMR (**Fig. 8**). The observation that this activity depends on expression of FZD_5 supports the intrinsic ability of this receptor to functionally interact with

heterotrimeric G proteins in living cells. However, the contribution of G proteins to the conformational changes observed in the FZD_5 FRET probe and other RET-based GPCR probes is less clear. Everything from no involvement to a direct involvement to extended kinetics of the agonist-induced response have been observed using prototypical GPCRs; thus, complicating interpretation of RET changes beyond ligand binding (Kauk and Hoffmann, 2018). Taken all together, these findings highlight the conformational diversity of GPCRs resulting from ambiguous cellular complexities like stoichiometry and equilibria that still require careful dissection – especially in the case of FZD_5 for which this has not yet been studied – in order to ascertain what drives a particular conformation and how that relates to receptor output in terms of signalling. Nevertheless, the concentration-response data presented in **Paper II** represents the first time – to our knowledge – that comparative pharmacology has been used in the context of WNT/FZD signalling in order to link ternary complex formation to downstream readouts.

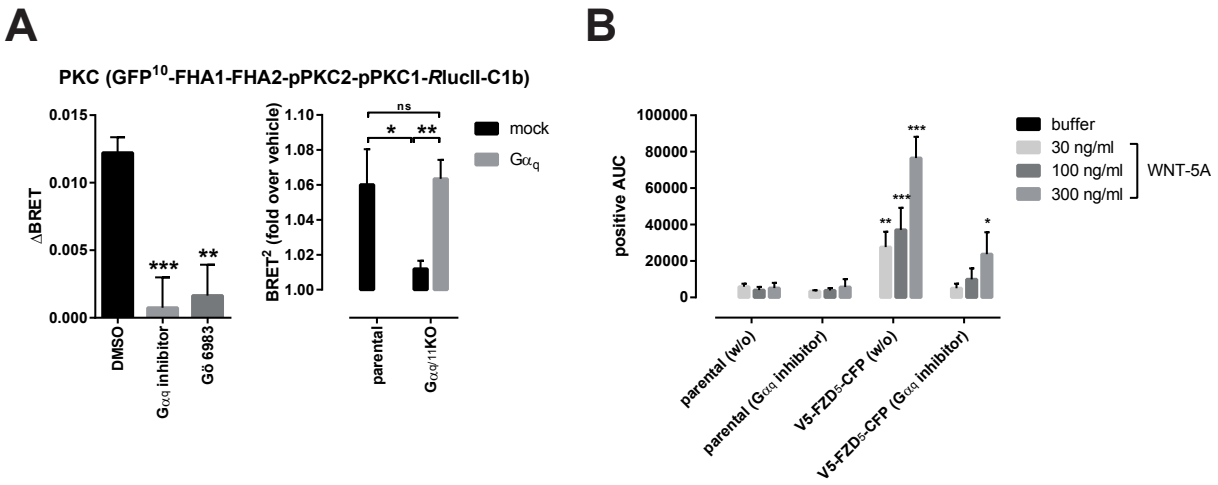

Fig. 8. FZD_5 utilizes endogenous Gq to activate downstream effectors and mediate whole-cell changes. (**A**) HEK293 cells expressing the PKC biosensor and FZD_5 were stimulated with WNT-5A in the presence or absence of inhibitors of $G\alpha_q$ or PKC proving that PKC is both active and downstream of $G\alpha_{q/11}$. A similar setup was used in $G\alpha_{q/11}$KO cells to confirm that the PKC response was exclusively dependent on $G\alpha_q$. (**B**) DMR experiments on HEK293 cells that expressed or did not express FZD_5 revealed that cells responded to WNT-5A in a FZD_5- and $G\alpha_q$-dependent manner.

4.4 IDENTIFICATION OF A MOLECULAR SWITCH IN CLASS F RECEPTORS

The absence of conserved motifs pertinent to Class A receptor activation has long been an argument against the GPCR nature of Class F receptors (Angers and Moon, 2009). This is in stark contrast to Class B/C receptors, which have evolved different motifs to arrive at a functionally equivalent signalling outcome – GEF activity. Disruption of the ionic lock or polar network in Class A and B receptors respectively is an integral step in receptor activation (Ballesteros et al., 2001; Liang et al., 2018b). An equivalent motif was recently identified in cholesterol- and cyclopamine-bound SMO structures without any insight into its functional relevance (Huang et al., 2016). Given the relevance of FZDs and SMO in cancer, we hypothesized that mutated residues important in Class F receptor activation would have a higher frequency in cancer patients compared to the general population (**Paper III**). Based on this rationale, we identified an arginine/lysine in position 32 on TM6 that formed hydrogen

bonds with the oxygen atoms of the TM7 helical backbone and π-cation interactions with the side chain of the conserved tryptophan in position 55 of TM7 – the same residues identified in the aforementioned study. In line with the genomic analysis of these patient datasets, structural analysis and molecular dynamics simulations confirmed the propensity of the hydrophobic alanine substitution in position 6.32 to produce a more open conformation. The outward movement of TM6 observed in the ligand-bound SMO structures and the loss in FRET observed upon WNT addition to cells expressing the FZD_5 sensor in **Paper II** collectively point to a molecular switch in Class F receptors that confers a conformational change in an agonist-bound state. Focusing on FZD_6, it became clear that the R416A mutant conveyed a higher ligand-induced activity and greater constitutive activity compared to the wild-type receptor in terms of calcium mobilization and phosphorylation of ERK1/2 respectively. Whereas there was a clear increase in the ability of FZD_6 to signal through heterotrimeric G proteins upon disruption of the molecular switch, the receptor was no longer capable of interacting with or inducing the phosphorylation signature on DVL that is characteristic of WNT pathway activation. In order to address this inherent bias, we performed concentration-response experiments with conformational sensors of the active GPCR state known as mini G (mG) proteins. Originally engineered to stabilize the active state of several GPCRs for the purpose of crystallization, they have recently been repurposed as cytosolically expressed BRET sensors that translocate to the membrane upon receptor activation (Carpenter et al., 2016; García-Nafría et al., 2018; Tsai et al., 2018; Wan et al., 2018). The shift in agonist potency observed for all representatives of the homology clusters of the Class F in the presence of mG protein follows the ternary complex model for GPCRs whereby the G protein can allosterically affect the receptor in such a way that confers increased affinity for the agonist (De Lean et al., 1980; Rasmussen et al., 2011a) (**Fig. 9**).

One of the caveats to this particular BRET-based setup is the dependency on direct RET between the luciferase-tagged receptor and the Venus-tagged mG protein. Consistent with the division of FZDs and SMO into their respective homology clusters, the length of the C-tail varies considerably and by consequence, the distance between the luciferase and the Venus as well as the dipole orientation can affect the RET efficiency and ultimately the apparent efficacy of the interaction. For this reason, it is impossible to compare different Class F members with each other and with other GPCRs in their ability to couple to mG proteins in this experimental paradigm. One possibility to circumvent this problem would be to use BRET sensors of G protein activation where the Gα subunit acts as a donor for the acceptor Gγ subunit. This approach was successfully used for FZD_5 in **Paper II** where the dynamic window displayed a marked difference compared to the response observed in the mG protein setup. In short, while we can readily detect a difference in agonist potency between wild-type and mutant receptors, other assays would be necessary to measure G protein coupling in the absence of an agonist-induced increase in BRET (e.g. wild-type FZD_7).

The choice of ligand should also be considered when probing for the active state of a GPCR. In **Paper III**, we limited the study to WNT-5A when looking at FZDs and Smoothened agonist (SAG) when looking at SMO. SAG is an obvious first choice since it is a synthetic small

molecule ligand that has been co-crystallized with SMO (Wang et al., 2014). However, the interaction profile between the 19 WNTs and 10 FZDs is less clear and largely based on binding experiments with recombinant CRD (Dijksterhuis et al., 2015). Therefore, we cannot say with certainty whether WNT-5A was a partial agonist or a full agonist in mG protein recruitment for the selected FZDs in the study.

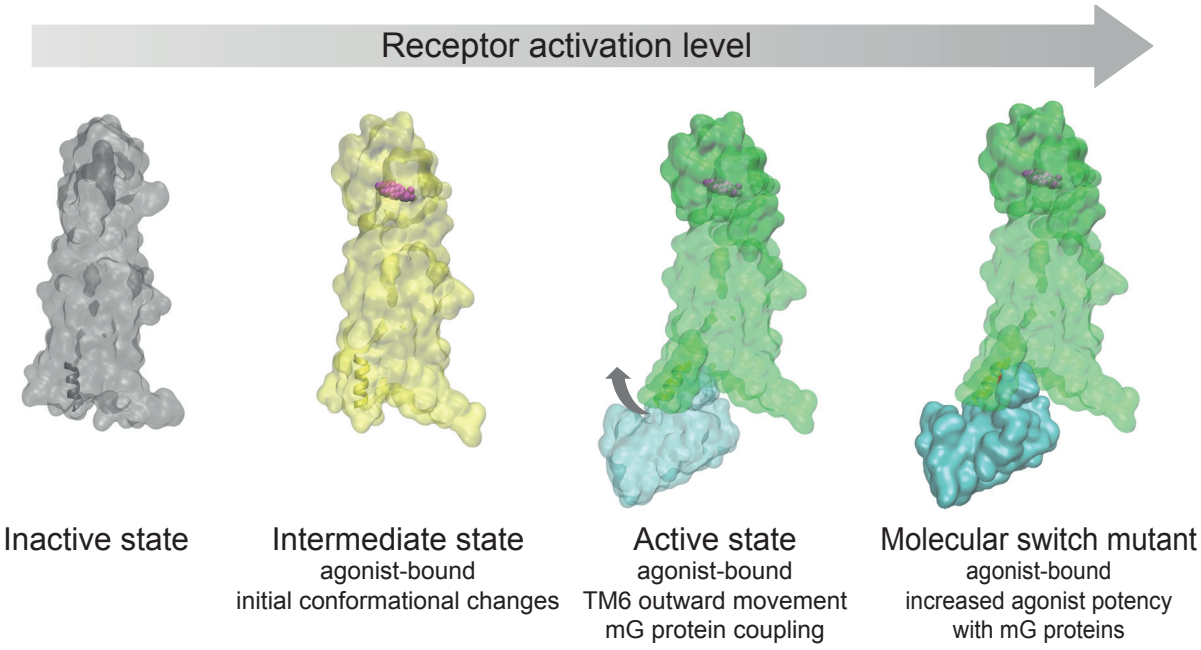

Fig. 9. Class F receptor activation. Different activation states of Class F receptors in the presence and absence of receptor-activating ligands, mG protein or molecular switch mutation.

4.5 USING SMO AS A BASIS FOR TARGETING FZDs PHARMACOLOGICALLY

Our structural insight into the activation mechanisms of Class F receptors remains limited despite advances in obtaining atomic resolution structures. Multidomain, ΔCRD and recombinant CRD structures in complex with diverse ligands have been solved; however, there is no indication how a two-component complex leads to receptor activity. SMO ligands have been crystallized bound to the CRD and the more traditional GPCR binding pocket in the TMD. Current dogma based on the FZD_8-CRD/WNT-8 crystal structure places the WNT binding site on the CRD (Janda et al., 2012). Norrin and *Clostridium difficile* toxin B have also been shown to interact with the CRD underlining its importance in the FZD binding mode. Conclusions drawn from the recent crystal structure of apo-ΔCRD-FZD_4 have reinforced the idea that FZDs behave differently from prototypical GPCRs. FZD_4 has a narrow, hydrophilic pocket in the transmembrane domain and a short TM6 that is tightly packed with the rest of the helical bundle (Yang et al., 2018). However, these findings fall short in explaining our findings in **Paper II** and **Paper III**. As exemplified by FZD_5 in **Paper II**, the differences in FRET upon agonist binding reflect conformational changes consistent with prototypical GPCR activation and inconsistent with dynamic kinks in TM7. Along the same line, all representative FZDs where the molecular switch had been mutated in **Paper III** displayed a shift in agonist potency in the

presence of mG protein reflecting changes in the agonist binding site that cannot exclusively be explained by WNT binding to the CRD. In other words, the thumb and index finger of WNT grasping the CRD cannot explain changes in the transmembrane bundle that would result in the observed conformational changes in either TM6 (as described for prototypical GPCRs) or TM7 (as proposed for FZD4). One possibility that requires experimental validation could be an analogous multi-step binding mode as described for Class B receptors (Schwartz and Frimurer, 2017). In the absence of agonist-bound full-length FZD structure, evidence is mounting that FZDs can be targeted via the traditional heptahelical ligand binding pocket. In **Paper III**, we further characterized the effect of small molecule SMO ligands that exert their effects on the 7TM core on the SMO Arg$^{6.32}$ mutant. Treatment of cells expressing the SMO Arg$^{6.32}$ mutant with cyclopamine-KAAD reversed the potency of SAG to values comparable to wild-type SMO (**Fig. 10**). Given the conserved activation mechanism in Class F and the finding that intracellular transducer binding can allosterically affect agonist affinity, this provides a proof-of-principle that FZDs can be drugged in a similar way to SMO – opening the door to new treatments targeting WNT/FZD signalling in disease.

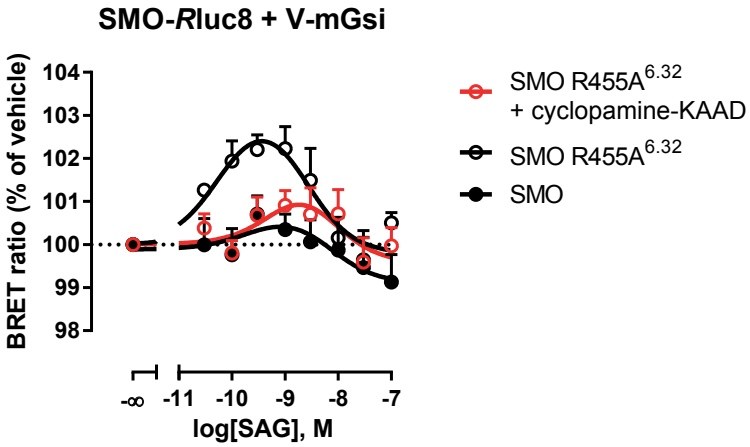

Fig. 10. Agonist potency shift of SMO R455A$^{6.32}$ can be reversed. SAG-induced SMO R455A$^{6.32}$ recruitment of mGsi is sensitive to inverse agonist suggesting that a similar small molecule approach could be used to target FZDs of equivalent mutations in disease.

5 CONCLUSION AND FUTURE PERSPECTIVES

The processes that guide the zygote to transition into a developing embryo are governed in large part by WNT and Hh signalling. Throughout gestation and into adult life, these signalling pathways are tightly regulated to ensure the physiological well-being of an individual. This molecular regulation is complex, orchestrated and fine-tuned during development beginning at the subcellular level to drive cell fate determination and organogenesis. Despite being conserved from worms to mammals, the complexity has increased over millions of years of evolution. In humans, there is a considerable disease burden due to misregulation of these pathways at the genetic and molecular levels. For this reason, there is a social demand for therapies that will increase life expectancy and improve its quality.

More than 30 years of WNT and Hh signalling research has answered many pertinent questions, but left us with several times as many more. One of the key unanswered questions revolves around the structural changes that occur in FZDs and SMO to induce pathway activation. The papers presented in this thesis aim to understand the dynamic, structural changes that reflect the switch from an inactive receptor to an active and transducer-bound Class F receptor. Their findings are highlighted here:

Paper I

- FZD_6 – one of 11 Class F paralogues – forms dimers in living cells
- FZD_6 dimerization is sensitive to WNT stimulation
- The dimer interface of FZD_6 is formed by TM4/5
- FZD_6 dimers are the inactive, ligand recognizing species
- Ligand-induced production of monomers results in receptor activity

Paper II

- WNT-5A stimulation of FZD_5 results in a conformational change
- FZD_5 forms an inactive state complex with $G\alpha_q$
- WNT-5A stimulation of FZD_5 leads to the activation of $G\alpha_q$, production of DAG, mobilization of intracellular Ca^{2+}, activation of PKC and whole-cell activation
- Pancreatic cancer cells – that functionally rely on FZD_5 for proliferation – are sensitive to $G\alpha_q$ inhibition

Paper III

- $R^{6.32}$ is the most prevalently mutated residue in cancer compared to population-level variation
- Network of residues between TM6/7 constitute a molecular switch
- Disruption of the molecular switch leads to higher agonist-induced and constitutive activity of FZD_6
- Analysis of the ability of FZD_5 and FZD_6 to signal through DVL reveals that mutagenesis of the molecular switch introduces receptor bias that feeds into pathway selectivity

- Conformational sensors of the active G protein-bound receptor state confirm that Class F receptor mutants are biased towards G protein signalling conferring higher agonist potency – indicative of a high affinity ternary complex
- Inverse agonist treatment of mutant SMO demonstrates the reversibility of this phenotype and the applicability of similar drug design to FZDs

The complexity of these signalling pathways has been a major roadblock for drug discovery efforts. Because WNT and Hh signalling are integral to development and adult tissue homeostasis, the issue of safety in the context of treatment has become a central concern (Kahn, 2014). To reiterate, the aim of this thesis is to better understand Class F receptor activation with the hope that we can translate mechanistic findings to the clinics. However, these findings are limited to basic pharmacology and a subset of human tumours. For example, there is no logic to targeting WNT receptors in the treatment of *APC* mutant gastro-intestinal tumours due to an etiology that is receptor-independent (Minde et al., 2011). With that in mind, multiple points of intervention are possible and under investigation in the treatment of diseases with overactive WNT/Hh signalling at the cell surface. A prime example comes from ring finger protein 43 (*RNF43*)-mutant pancreatic adenocarcinomas for which hypothetical points of intervention include Porcupine (PORCN), GPR177/Wntless, WNTs, FZDs which cannot be ubiquitinated by RNF43 and $G\alpha_q$.

Antibodies that target the FZD CRD and chimeric CRD-Fragment crystallizable region (Fc) fusions have garnered some attention for their progression into clinical trials for the treatment of various cancers. However, vantictumab and ipafricept were both discontinued at the end of 2017 begging the question whether antibody-based antagonism of WNT signalling to inhibit protein-protein interactions (PPIs) makes sense from a strategical standpoint. Studies based on the crystal structure of WNT-8 in complex with the FZD_8 CRD have driven home the message that blocking the CRD will prevent WNT binding. What these studies have failed to consider is evidence of a multi-step binding mode in Class B receptors, a pharmacophore in the 7TM bundle of the Class F member SMO and evidence that we cannot achieve sufficient biological selectivity to target specific FZD paralogues. Nevertheless, from a development perspective, antibodies are advantageously more successful; they have a longer half-life *in vivo* and higher affinity and potency through specific selectivity – of which the latter is thus far unachievable among FZD CRDs. On the downside, they are more expensive, cannot penetrate cells and as foreign proteins, they are intrinsically immunogenic (Imai and Takaoka, 2006). Currently, several camelid-based antibodies called nanobodies have worked their way into clinical trials. Nanobodies are single domain fragments that maintain all of the advantages of conventional antibodies while boasting the ability to distinguish protein conformations and access cavities or clefts that larger antibodies would otherwise not be able to target (Steyaert and Kobilka, 2011).

Alternative molecular targeting strategies are already in place for SMO as evidenced by the inhibitor vismodegib – a small molecule inhibitor that binds the 7TM bundle and is currently being used to treat basal cell carcinoma. Contrary to antibodies, small molecules are less

expensive, easier to administer and have the potential to penetrate cells. Small molecules are notoriously less specific and there is the risk for pharmacokinetic variability affecting bioavailability across individuals (Imai and Takaoka, 2006). In spite of this, small molecule targeting of SMO has been successful resulting in the development of both agonists and inverse agonists that bind SMO in a traditional GPCR manner.

From an inactive, tightly packed Class F transmembrane structure reinforced by receptor dimerization to disruption of a molecular switch in helices TM6/7 that leads to the outward movement of TM6 allowing for monomeric receptor engagement and activation of heterotrimeric G proteins, this thesis details the molecular events that entail Class F receptor activation. FZDs and SMO are dynamic proteins that respond bidirectionally to stimuli through conformational changes in their protein structure. The fundamental understanding that GPCRs are molecular machines whose activities are distinguished by their conformations has not been empirically applied to FZDs nor SMO. The work detailed in this thesis provides the foundation for future studies into Class F receptor activation that will dig deeper into the conformational landscapes of the receptors implicated in WNT and Hh signalling. More specifically, the methods used in this thesis to measure FZD and SMO activity begin to illuminate what was previously referred to as the "black box" of WNT and Hh signalling (Schulte, 2015).

This raises the following question: how can these findings and methods be used moving forward to screen for new drugs that can be used to treat diseases of aberrant WNT or Hh signalling?

The answer is multifactorial, but lies in 1) the scalability of the method – of which several in this thesis can be used in medium- to high-throughput screening, 2) an understanding of the target cell physiology and 3) a strategy to achieve paralogue selectivity and pathway specificity.

RET-based biosensors and label-free approaches like DMR in combination with the recently described $FZD_{1\text{-}10}^{-/-}$ mutant HEK293 cells (Eubelen et al., 2018) provide a logical starting point for systematically analyzing FZD paralogues. Although these cells do not adequately mimic the physiological context where the receptors are normally expressed, they are a practical system for characterizing FZD activation. Subsequent efforts will surely focus on contextualizing the effect of the drug in physiological relevant cell and model systems where intracellular effectors, trafficking properties and overall protein composition may differ.

The issue of subtype selectivity is not new to the GPCR superfamily. Muscarinic receptors – of which there are five paralogues M_1-M_5 – all bind and mediate acetylcholine signalling. This has led to the development of dualsteric ligands that bind both orthosteric and allosteric sites to achieve subtype selectivity (Antony et al., 2009). While this is obviously not an equivalent analogy given that 19 WNTs exist for 10 FZDs, it offers some idea for how antagonists or inverse agonists may be designed to eliminate poor paralogue selectivity.

In conclusion, FZDs and SMO are GPCRs that dynamically and functionally respond to stimuli to mediate the activation of intracellular transducers including heterotrimeric G proteins. More

work needs to be done to address the drawbacks and failures associated with current drug treatments targeting diseases of WNT signalling. Integration of the kinetic responses that are fast and transient at the plasma membrane in a physiologically relevant manner with the downstream signalling cascades that lead to transcriptional regulation will facilitate these efforts and hopefully bring relief to those afflicted by disease.

6 SAMMANFATTNING

Vår genetiska uppsättning består av tusentals gener som kodar för de proteiner som är involverade i alla aspekter av våra cellfunktioner. Proteiner är molekylära maskiner som dynamiskt och strukturellt svarar på stimuli. Våra celler besitter förmågan att känna av sin omgivning och att reagera följaktligen med hjälp av speciella proteiner kallas receptorer. Dessa ligger inbäddade i det cellulära membranet och binder till extracellulära molekyler kända som ligander. Receptorernas karaktäristiska förmåga att specifikt interagera med och påverka strukturella ändringar hos partnerproteiner utgör basen för signalkaskaden som reglerar cellulära processer. Vid sjukdomstillstånd kan genetiska mutationer resultera i ändringar i proteinstruktur som kan påverka receptorernas tertiära och kvartära struktur i proteinkomplexet. Detta kan resultera i drastiska effekter på proteinfunktion och så småningom cellens förbestämda öde.

Användningen av animaliskt och växtbaserade produkter inom den moderna medicinen har lett till utvecklingen av farmakologin – läran om hur levande organismer påverkas av läkemedel. Tekniker för märkning av receptorer och molekylär kloning har gjort det möjligt att fastställa att flera av dessa molekyler agerar på receptorfamiljen vid namn G proteinkopplade receptorer (GPCRer). Vår kunskap gällande receptorernas strukturella ändringar vid ligandinbinding har bidragit till utvecklingen av nya läkemedel med bättre specificitet och färre biverkningar.

Medan vår kunskap relaterad till prototypiska GPCRer som adenosinreceptorer och opioidreceptorer har ökat de senaste åren har kunskapen kring deras avlägsna kusiner, kända som Frizzleds (FZDs) och Smoothened (SMO), dock lämnats utan möjligheter för utvärdering av deras GPCR egenskaper. FZDs interagerar med lipoglykoproteiner tillhörande WNT familjen medan SMO regleras indirekt av Hedgehog (Hh) för att samordna viktiga processer under den embryonala utvecklingen och homeostasen. Fel i WNT/FZD och Hh signaleringen leder till patogena tillstånd för vilka det oftast finns begränsade möjligheter för behandling. Med hänsyn till WNT signalering beror bristen på effektiva läkemedel som riktar sig mot FZDs främst på kunskapsbrist orsakad av den aktuella dogman som framhäver bildandet av proteinkomplex före strukturella ändringar involverade i proteinaktivering.

Upptäckterna sammanställda i **artiklarna I** till **III** bistår med strukturella insikter i de mekanismer som ligger till grund för FZD/SMO aktivering. En kombination av genomiska, biofysiska samt biokemiska tillvägagångssätt har använts för att avslöja detaljer kring bildandet av FZD/SMO komplex och den strukturdynamik som uppstår då WNT binder och initierar full receptoraktivitet och nedströms signalering. Detta har betydelse för utveckling av effektiv behandling vid sjukdomstillstånd där FZDs och SMO kan användas som målmolekyler. Ytterligare karaktärisering av dessa receptorers konformationslandskap kommer att underlätta framtida försök för läkemedelsutveckling.

7 ACKNOWLEDGEMENTS

To all of my non-scientist family and friends, you might be wondering what factors contributed to this nearly lifelong machoistic journey of academic and scientific study. Obviously, if you do not love what you do, then you are in the wrong field and should reconsider your career path. But personally speaking, I developed a strong desire to dig deeper into the unknown and challenge myself to understand the things in life that we cannot see (without the right instruments). With all that said, this journey would have been much lonelier and far more difficult – if not impossible – without the following people…

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